# Chapter 7

# NMR Studies on the Conformation of Polyflavanoids and their Association With Proteins

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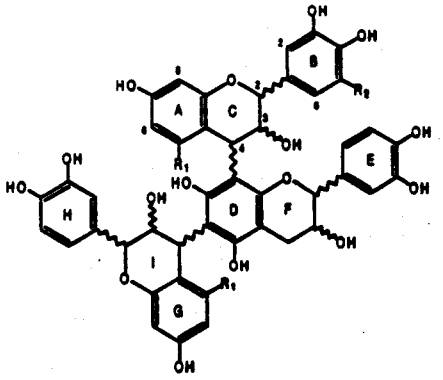
## INTRODUCTION

Polyflavanoids (also named condensed tannins or proanthocyanidins) make up approximately half of the dry weight of most commercial tree barks, are often found in even higher concentrations in nut shells, and are important constituents of the leaves of plants. (*18,27*) The polyflavanoids rank second in abundance after lignin as a source of renewable phenolic materials. Most of their commercial and ecological significance centers on either their propensity to form complexes with proteins or on their potent anti-oxidant properties (*21,42*).

Because it is believed that the association of polyflavanoids with proteins is dictated by the shape and flexibility of these molecules, (*15*) we have undertaken an effort to try to define the conformational dynamics of polyflavanoids and to learn more about the interaction of polyflavanoids with proteins. (*11,19,35,39*) Our approach has been centered mainly on NMR experiments to obtain the necessary physical data to evaluate the results of computational chemistry. (*22,41*) Both NMR instrumentation and computational chemistry software are advancing at a fast pace and increasingly offer avenues to solution of questions surrounding tannin/protein interactions.

The conventional systems for naming and numbering the rings in a trimeric proanthocyanidin are shown in Figure 1.

**Figure 1: General Structural Features of Plant Polyflavanoids.** *(18,27)*




Note that each chain extender unit has three chiral centers so the absolute stereochemistry at each center must be defined. The structural features that relate to their shape and flexibility center on:

1) heterocyclic ring stereochemistry;
2) configurational isomerism due to heterogeneity (or homogeneity) in the location of the interflavanyl bond;
3) rotational isomerism due to restricted rotation about the interflavanyl bond; and
4) conformational isomerism due to flexing of the pyran ring.

NMR experiments provide access to resolution of the questions surrounding all of these issues. While an understanding of the conformational dynamics of these compounds is essential, it is also important to relate that information more directly to questions of how the polyflavanoids interact with proteins. Here too, NMR experiments provide critical physical data to better understand the interactions between polyflavanoid and polypeptides. NMR experiments can reveal intermolecular interactions either through changes in chemical shifts or through nuclear Overhauser effects. We have concentrated our work on the association of oligomeric flavanoids with oligomeric peptides in which both NMR data and computational chemistry approaches are manageable within the context of the instrumentation available to us. The results obtained from study of oligomers should provide a basis for understanding the behavior of the higher polymers.

Some of the NMR experiments we have used to address these problems are described here with the intent of demonstrating the wide range of experiments that have been required to solve various problems. By necessity, this chapter concentrates on our own work and does not pretend to be a thorough review of the literature which is vast because most work on the chemistry of these compounds involves NMR experiments. The intent is to provide the reader with some examples how NMR experiments have been used to solve a variety of problems.

## Heterocyclic Ring Stereochemistry

In constrast to lignin, the polyflavanoids are comparatively small molecules and, those within a plant species and tissue, are of a comparatively specific uniform structure (Figure 1). A typical condensed tannin isolate shows a broad distribution of molecular weight but with a number average molecular weight of only about 3,000-5,000 (between 10 and 17 flavan units) (*7,26*).

The interflavanyl bond in the 5,7-dihydroxyflavans, such as the procyanidins and prodelphinidins present in most commercially important tree barks, can be cleaved under mild acid conditions and the constituent units in the higher polymers can be established by analysis of the cleavage products. (*5,32*) The interflavanyl bond in the "5-deoxy" profisetinidins and prorobinetinidins, such as in wattle tannins, resists cleavage under mild acidic or alkaline conditions but, fortunately, these extracts tend to be dominated by trimers and tetramers so most structural features can be defined by NMR experiments.

The absolute stereochemistry at C-$2_C$ is usually consistent within the chain extender units. Compounds with 2*R* absolute stereochemistry predominate being present in the vast majority of the hundreds of plants examined to date. (*18,27*) However, natural compounds with 2*S* absolute stereochemistry do exist and are represented by the commercially important Quebracho (*44*) and Rhus spp. (*4*) tannins. The absolute stereochemistry at C-$2_C$ in the terminal unit is usually, but apparently not always, (*44*) similar to that in the chain extender units. Within the 5-deoxy profisetinidins and prorobinetinidins in which the interflavanyl bond resists cleavage, complete proof of the absolute stereochemistry requires the synthesis of isomers of known stereochemistry. (*32,37*)

Once the absolute stereochemistry at the C-$2_C$ or C-$4_C$ positions is established, the relative stereochemistry at the pyran ring chiral centers can usually be deter ined by $^1$H NMR experiments from the heterocyclic ring proton coupling (Table 1). (*24*)

**Table 1: $^1$H NMR Coupling Constants of Methyl Ether Acetate and Peracetate Derivatives of Procyanidin Dimers.*(24)***

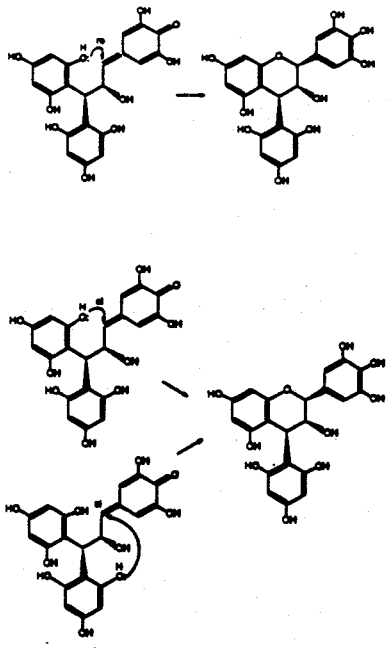
| Stereochemistry | Methyl Ether Acetate | | Acetate | |
|---|---|---|---|---|
| | $J_{2,3}$ | $J_{3,4}$ | $J_{2,3}$ | $J_{3,4}$ |
| 2,3-*trans*-2,3-*trans* | 10 | 8.7-9.5 | 9.6-10.0 | 9.0-9.6 |
| 2.3-*trans*-3.4-*cis* | 9.5 | 5.5 | 9.6 | 6.5 |
| 3,4-*cis*-3,4-*trans* | 0-1.7 | 2.0-2.2 | 1 | 1.5 |
| 2,3-*cis*-3,4-*cis* | 1 | 4.7-4.9 | 1 | 5.5-6.0 |

Evidence for either 2,3-*cis* or 2,3-*trans* relative stereochemistry can also obtained from the C-$2_C$ chemical shift in a $^{13}$C NMR experiment. (*28*) However, care must be exercised in the interpretation of proton coupling constants as described more fully in discussion of the 2,3-*cis*-3,4-*trans* and 2,3-*cis*-3,4-*cis* isomers as well as difficulties associated with conformational flexing of the pyran ring reviewed below.

In the 5-deoxy profisetinidins and prorobinetinidins such as those in wattle tannins, the chain extender units are either 2,3-*trans*-3,4-*trans* or 2,3-*trans*-3,4-*cis* relative stereo-chemistry. (*30*) Profisetinidins that are of 2,3-*cis*-3,4-*trans* stereochemistry are extremely rare, the tannins from *Pithecellobium dulce* (Madras thorn) bark being the best source known of that stereochemistry to date. (*36*) Within the 5,7-dihydroxyflavans typical of most tree bark tannins, the chain extender units are predominantly of 2,3-*cis*-3,4-*trans* relative stereochemistry. (*18,27*) The terminal unit is typically 2,3-*trans* as in (+)-catechin. This monomeric flavan-3-ol is also normally found in plant extracts. (*27*)

The isolation of the 2S all-*cis* *ent*-epicatechin-(4β→2)-phloroglucinol (*37*) from acid-catalyzed cleavage of 2R prodelphinidins from pecan nut pith tannins in the presence of phloroglucinol as a capture nucleophile is an example of the care that must be taken in assigning the stereochemistry of these compounds (Figure 2).

**Figure 2: Origins of Epigallocatechin-(4β→2)-Phloroglucinol and *ent*-Epigallocatechin-(4β→2)-Phloroglucinol from Cleavage of Pecan Pith Tannins.*(37)***



It has long been assumed that cleavage of the interflavanoid bond of procyanidins and prodelphinidins can be achieved with retention of configuration at C-$2_C$ when reacting these compounds with capture nucleophiles such as thiols or phenols such as phloroglucinol. (*5*) However, two epigallocatechin-(4→2)-phloroglucinol adducts with similar proton coupling constants of $J_{2,3} = < 1$ $J_{3,4} = 1.4$ and $J_{2,3} = 1.5$, $J_{3,4} = 2.0$ Hz (as measured from their methyl ether acetate derivatives) were obtained from reactions that were prolonged to 24 and 48 hours at 100 °C in an attempt to increase the yield of flavan-(4→2)-phloroglucinol cleavage products from pecan pith tannins.

Circular dichroism (CD) measurements showed that the absolute stereochemistry at C-$4_C$ was 4*S* (a 4β→2 linkage) in both products. (*37*) While evident in the study of other compounds in which H-$2_C$ and H-$4_C$ are both axial, no NOE correlation was observed between H-$2_C$ and H-$4_C$ in either NOESY or NOE difference experiments on either compound. However, the NOESY experiment did show a correlation between the methoxy protons of the pyrogallol B-ring and the phloroglucinol D-ring in one of these products. When coupled with the CD results, the compound showing a strong NOE cross peak must be of a 2*S*, 3*S*, 4*S* (all-*cis*) stereochemisty. As would be expected, no NOE was seen between H-$2_C$ and H-$4_C$ or between the B- and D-rings in

the 2*R*, 3*R*, 4*S* isomer that would be expected from a 4β substitution of the carbocation derived from 2,3-*cis* constituent units in the polymer.

In the proof of the structures of the novel 2,3-*cis* profisetinidins isolated from Guamuchil,(*36*) the relative stereochemistry of the chain extender and terminal units was obvious, from $^{1}H$ and $^{13}C$ NMR spectra. The configuration at C-4 of the chain extender units was apparent from the CD spectra and consistent with 4*S* absolute stereochemistry. The most difficult challenge came when the absolute stereochemistry of the 2,3-*cis* and 2,3-*trans* terminal units had to be proven. Because it was not possible to cleave the interflavanyl bond under mild conditions, synthesis of dimers and trimers with 2*R* and 2*S* absolute stereochemistry in the chain extender and more importantly also the terminal units from precursors of defined absolute stereochemistry was required. (*36*) The $^{1}H$ NMR spectra of many of these compounds exhibited severe broadening due to slow rotation at ambient temperature (see discussion below) but first order spectra were obtained when spectra were recorded at 343 °K.

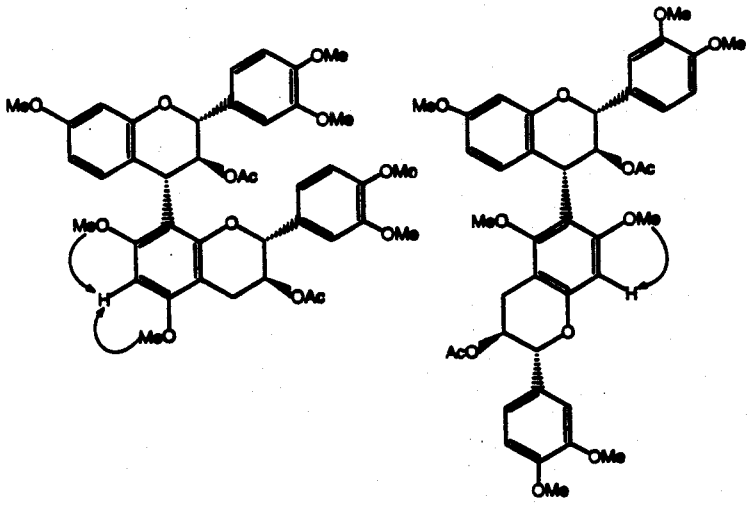
**Configurational Isomerism at the Interflavanyl Bond**

Regio-isomerism involved with the location of the interflavanyl bond in dimeric procyanidins was first described by Weinges (*46*) following from the strong effort made by Freudenberg, Mayer, and Weinges and their coworkers at Heidelberg through the 1960s. That work is summarized in a review (*45*) that still is most valuable. Since the late 1960's, there has been explosive growth in our understanding of oligomeric proanthocyanidins driven by the works of Roux, (*31-33*) Haslam, (*14,15*) and many other laboratories around the world.

In studies of the procyanidins in southern pine bark, three trimers that differed in the location of the interflavanyl bond: a) epicatechin-(4β→8)-epicatechin-(4β→8)-catechin. b) epicatechin-(4β→8)-epicatechin-(4β→6)-catechin and c) epicatechin-(4β→6)-epicatechin-(4β→8)-catechin were isolated and partial thiolytic cleavage of the polymer gave dimeric cleavage products representing both regio-isomers.(*20*) It is interesting to reflect on the considerable work with unpleasant thiols that was required to synthesize the different dimer thioether derivatives and how simple the proof of structure would have been if we had access to HMQC and HMBC experiments then. Despite the progress on the chemistry of procyanidins, the question of whether plants produce linear all-(4→8) linked procyanidins and heterogeneity in the location of the interflavanyl bond in procyanidins is due to rearrangement in the isolation of these compounds or if these compounds are produced by plants with a mixture of (4→6) and (4→8) interflavanyl bonds is still not resolved.

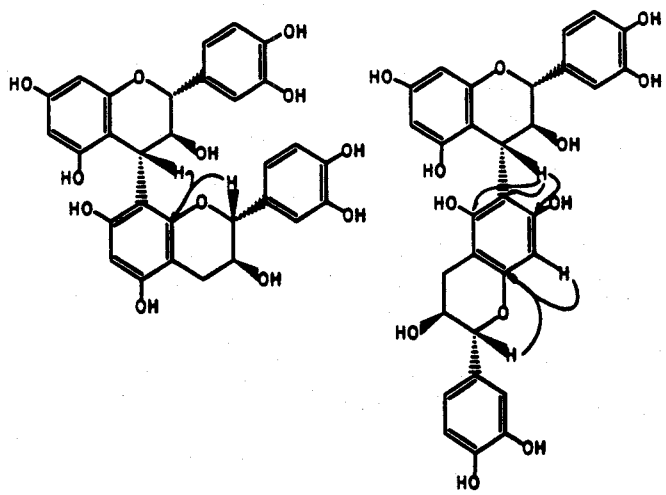
When examined carefully, the differences between these structures are slight and near-symmetry presents a challenge in distinguishing between the procyanidins and prodelphinidins with either (4→6) or (4→8) interflavanoid bonds using simple $^{1}H$- or $^{13}C$-NMR experiments. One approach to resolution of questions about interflavanyl bond location is to make the methyl ether or methyl ether acetate derivatives and to use methoxy NOE experiments. (*34*) Both the MeO-$5_A$ and MeO-$7_A$ methoxy groups are correlated with H-$6_A$ whereas only the MeO-$7_A$ will see H-$8_A$ permitting differentiation of the A- and D-ring methoxyls in both structures. In compounds with a (4→8)-linkage, both the MeO-$5_D$ and MeO-$7_D$ methoxy groups will show correlation to H-$6_D$. By contrast, in compounds with a (4→6)-linkage, only a correlation between MeO-$7_D$ and H-$8_D$ is observed (Figure 3).

**Figure 3: Methoxy NOE Experiments Define Interflavanyl Bond Location In Methylated Derivatives.*(34)***




We have also used both HMBC and COLOC experiments to resolve the question of interflavanyl bond location in the mixture of rotational isomers seen in 2,3-*trans* procyanidins in their free phenolic form. (*17*) In the (4→8)-linked structure, long-range correlations can be seen from H-$4_C$ and H-$2_F$ to C-$10_D$. However, in the (4→6)-linked structure, there are long range correlations between H-$4_C$ and C-$5_D$ as well as C-$7_D$ and these carbon signals can be distinguished from C-$10_D$ by its correlation with H-$8_D$ and H-$2_F$ (Figure 4).

**Figure 4: COLOC and HMBC Experiments Define Interflavanyl Bond in Phenolic Forms of Proanthocyanidins.** *(17)*

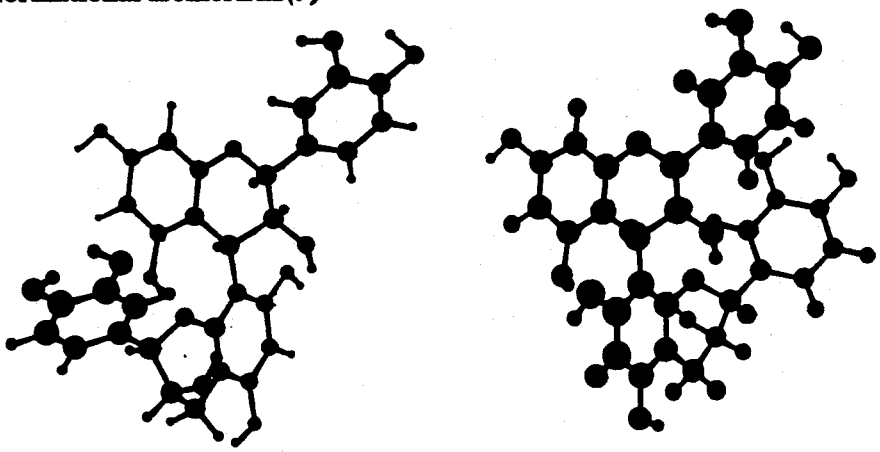


Balas and Vercauteren (*1,2*) have used a similar approach to define the location of the interflavanyl bonds in peracetate derivatives of dimeric and trimeric procyanidins.

**Rotational Isomerism**

Hindered rotation about the interflavanoid bond (8) results in doubling of the signals in the NMR spectra where rotation is restricted (Figure 5 ).

**Figure 5: Restricted Rotation about the Interflavanyl Bond Leads to Conformational Isomerism.** *(9)*

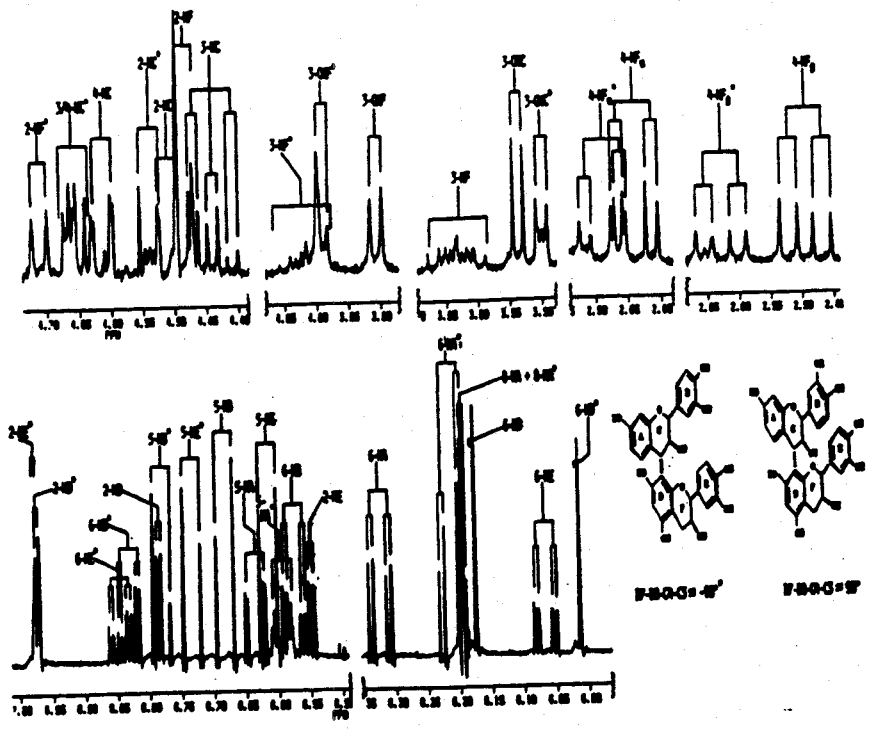


Severe broadening of the proton signals is seen where slow rotation occurs during the time frame of the NMR experiment. Fletcher (*5*) examined this phenomenon for the procyanidins that are most commonly found in commercially important tree barks. At ambient temperature, two sharp sets of signals due to each rotamer are seen in the proton spectra of the 2,3-*trans*-3,4-*trans* procyanidins such as catechin-(4α→8)-catechin whereas only broadened signals are seen for the 2, 3-*cis*-3, 4-*trans* isomers such as epicatechin-(4α→8)-catechin in which the lower unit is quasi-axial to the plane of the A and C ring of the upper unit. Even where rotation is fast enough to show a first order NMR spectrum, the presence of two preferred rotational isomers can be seen using time-resolved fluorescence decay analyses because of the much shorter time frame for those experiments (*3*).

A combination of COSY and NOE difference experiments was used in order to fully assign the $^1$H NMR spectra for each of two rotamers observed in the methyl ether acetate derivatives of a series of dimeric profisetinidins. (*34*) A COSY experiment permits one to assign the heterocyclic ring protons of the upper and lower units in each of the rotamers and long range correlations allow assignments of the methoxy protons as well as the B and E rings for each rotamer. Once the Ar-H and methoxy protons for each rotamer are assigned, the orientation of the two rotamers can be established. In the more compact rotamer in which the E- and F-rings are back behind the plane of the A- and C-ring, NOE between the MeO-$7_D$ and H-$4_C$ is evident. In the more extended rotamer in which the E- and F-rings extend out from the plane of the A- and C-ring, no correlation between these protons is observed.

Steynberg (*35*) used a similar series of NMR experiments in a conformational analysis of the profisetinidin dimer (-)-fisetinidol-(4α→8)-catechin in the free phenolic form. Here the $^1$H NMR spectrum shows two rotational isomers in approximately equal proportion (Figure 6).

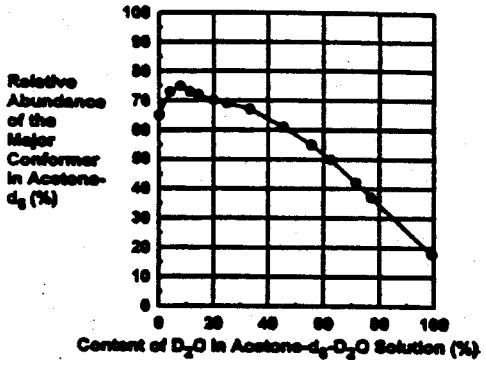
**Figure 6: The Proton NMR Spectrum of Fisetinidol-(4α→8)-Catechin Displays Two Rotational Isomers in Nearly Equal Proportion.** *(35)*



A combination of homonuclear J-Resolved, COSY, and HETCOR experiments provided most of the information needed to unequivocally assign the protons of the two rotamers. By working in an especially dry environment with extensively dried $d_6$-acetone, exchange of the hydroxy protons could be slowed sufficiently to allow hydroxy NOE experiments. NOE from OH-$7_D$ to H-$4_C$ in one rotamer and from OH-$7_D$ to H-$3_C$ in the other defined the conformations of the two rotamers. As was seen in the methyl ether acetates described above, the slightly preferred rotamer was the more compact one in which the E- and F-rings are back behind the plane of the A- and C-rings. Steynberg suggested on the basis of chemical shifts of the E-ring protons that the compact rotamer is heavily favored when this compound is dissolved in the biologically significant solvent, water (*35*).

Hatano (*17*) studied the effect of solvent on the rotational isomer preferences of the procyanidin dimers catechin-(4α→8)-catechin and catechin-(4α→8)-epicatechin and found a similar result with one predominant rotamer evident when dissolved in $D_2O$. When the proton spectrum of catechin-(4α→8)-epicatechin is recorded in $d_6$-acetone, the more extended rotamer is present at about 65% of the population. Addition of increasing amounts of $D_2O$ resulted in a gradual decline in the population of the extended rotamer until it represented only 20% when dissolved in $D_2O$ (Figure 7).

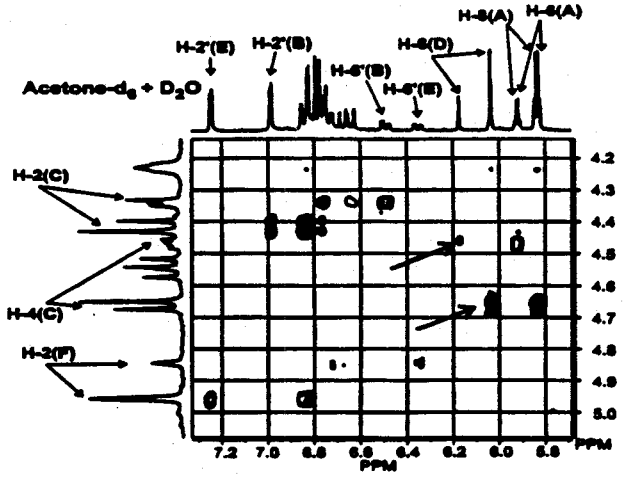
**Figure 7: Conformational Preferences of Dimers are Influenced Strongly by Solvent Composition.** *(17)*




Similarly, the extended form of the dimer catechin-(4α→8)-catechin represents about 40% of the population when dissoved in $d_6$-acetone and only signals representing the more compact rotamer are seen when dissolved in $D_2O$.

A long-range COSY experiment provided evidence for the orientation of the two flavan units in the two rotamers.(*17*) Strong cross peaks were seen between H-$4_C$ and both H-$6_A$ and H-$8_A$, analogous to allylic coupling where the overlapping of the C-H σ-bond and the π orbitals is maximized at a 0° dihedral angle. The H-$6_A$ and H-$8_A$ form an approximate 90° angle relative to H-$4_C$ in both rotamers and strong cross peaks to both are observed (Figure 8).

**Figure 8: A Long-Range COSY Experiment Provides Information on the Torsion Between Flavan Units of Dimers.*(17)***



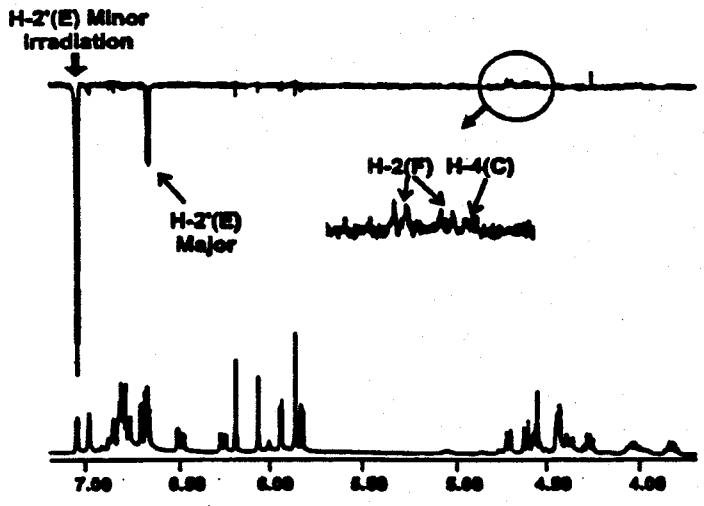

The important aspect of this experiment is that it provides a probe of the orientation of the terminal unit. For the dimer catechin-(4α→8)-epicatechin in $d_6$-acetone strong cross peaks are seen in the major rotamer between H-$4_C$ and H-$6_D$ indicating an angle between these protons of approximately 90°. By contrast, the minor rotamer showed only a very weak cross peak between H-$4_C$ and H-$6_D$ suggesting an angle deviating to 0° or 180°. Just the opposite situation was observed in study of catechin-(4α→8)-catechin in $d_6$-acetone. Here there was a strong cross peak between H-$4_C$ and H-$6_D$ in the minor rotamer and no correlation between those protons in the major rotamer. These results indicate that the angle between H-$4_C$ and H-$6_D$ is about 90° in the minor rotamer but that it is distorted more toward either 0° or 180° in the major rotamer.

The conclusions reached from the long range COSY experiments were supported by measurements of NOE difference and NOESY experiments. (*17*) Irradiation of H-$2_B$ resulted in a strong positive NOE with H-$4_C$ as well as a negative (conformational exchange) signal for H-$2_B$ of the minor rotamer in catechin-(4α→8)-epicatechin. By contrast, irradiation of H-$2_B$ of the minor rotamer of catechin-(4α→8)-catechin resulted in comparatively weak positive NOE with both H-$2_F$ and H-$4_C$ for the minor rotamer and a stronger negative (conformation exchange) signal for H-$2_B$ of the major rotamer.

It is important to recognize the dynamics involved in the conformations of these molecules. Even though two distinct rotational isomers are seen as sharp $^1$H NMR

signals in dimers such as the 2,3-*trans*-3,4-*trans* procyanidins, conformational exchange (*17*) is an important feature of the NOESY and NOE difference spectra obtained from these compounds (Figure 9).

**Figure 9: Conformational Exchange is a Prominent Feature of NOESY and NOE Difference Spectra Despite High Rotational Energy Barrier.** ***(17)***



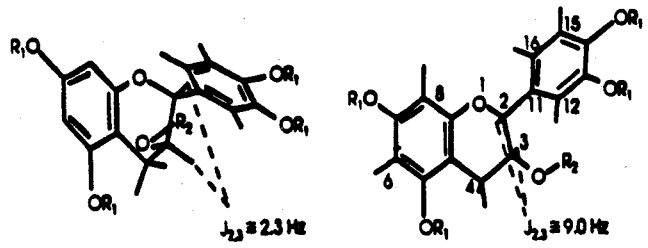

When examined using the MM2 forcefield, two rotational isomers with only small (ca. 3.7 kcal/mol) energy differences are seen in which the C-$3_C$-C-$4_C$-C-$8_D$-C-$9_D$ torsion angels center on +/- 90°. The flexibility in rotation is limited to a narrow range of +/- 75° to +/-104° for acceptable energy because of severe steric interactions. (*43*) The high energy barrier to rotation through 360° begs the question of whether the two "rotamers" correspond to conformations in which the lower unit is really either out from or back behind the plane of the A- and C-rings with conformational exchange through such high energy barriers. The same question arose from study of the conformation of the lower unit pyran ring through line shape analysis of the H-$3_F$ coupling as discussed below.

## Conformational Isomerism of the Pyran Ring

Considerable effort has been put to understanding of the conformational dynamics of the the pyran ring because observed couplings between H-$2_C$, H-$3_C$ and H-$4_C$ do not agree with values calculated for a low energy "half-chair" conformation in which the B-ring is *quasi*-equatorial to the plane of the A-C rings. For example, measured $J_{2,3}$ coupling for the 2,3-*trans* flavan-3-ol derivatives generally fall in the range of 6-7 Hz

in contrast to expected values of about 10 Hz. The observation that the pyran ring in the crystal form of the penta-acetate of (+)-catechin is in a "reverse half-chair" with the B-ring *quasi*-axial (*10*) prompted Porter (*29*) to suggest that the small coupling constants observed in NMR experiments result from averaging of the E-(B-ring equatorial) and A- (B-ring axial) conformers due to rapid conformational exchange in the time frame of the NMR experiment (Figure 10).

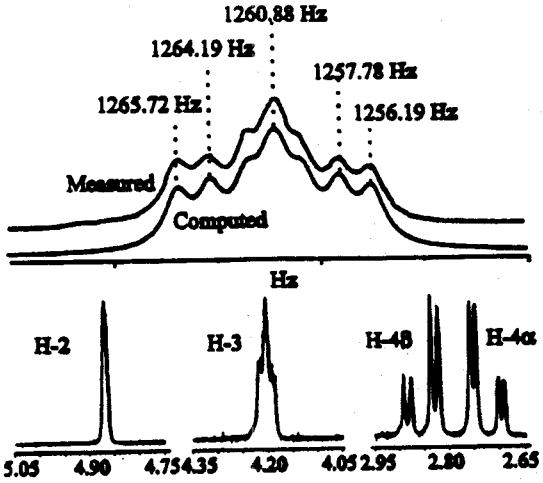
**Figure 10: Computations Show A- and E-Conformers of Similar Energy and Observed $J_{2,3}$ Coupling is Typically Intermediate Suggesting A- to E-Conformational Averaging in Monomeric Flavan-3-ol Derivatives.** ***(29,40)***




Evidence supporting this A- to E-conformational exchange in the 2,3-*trans* flavan-3-ol derivatives has been obtained in molecular dynamics computations (*6,12*) and as well as conformational search methods (*40*) including reasonably close approximations of the changes in heterocyclic ring coupling constants associated with variation in temperature. (*41*)

Similar studies on the 2,3-*cis* flavan-3-ol *ent*-epifisetinidol, (*39*) while consistent with the hypothesis of A- to E-conformational exchange, were not totally conclusive because H-$2_C$ appears as a broad signal with about 2Hz coupling. However, good estimates of the coupling constants for the heterocyclic ring could be obtained by line shape analysis of H-$3_C$ (Figure 11). (*22*)

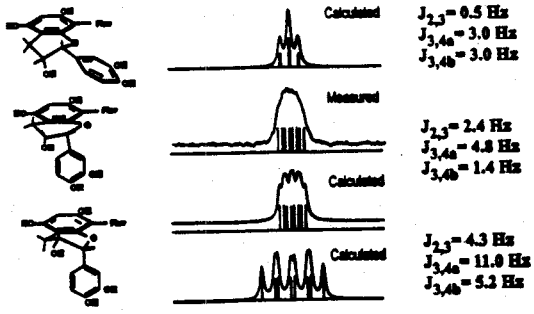
**Figure 11: Lineshape Analysis of H-3C Provides Access to all the Coupling Constants in the Pyran Ring of 2,3-*cis* Flavan-3-ols.*(19,22)***




The assignments given to H-$4_\alpha$ and H-$4_\beta$ in (-)-epicatechin (22) were later shown to be incorrect by a multi-NOE experiment (*9*) and should be reversed. The correct coupling constants are: $J_{2,3} = 1.6$; $J_{3,4\alpha} = 3.3$; $J_{3,4\beta} = 4.5$ Hz. These coupling constants are also consistent with the results of a GMMX global search computation which indicated that a Boltzmann average of the A- and E-conformer populations could suitably predict the observed coupling constants.

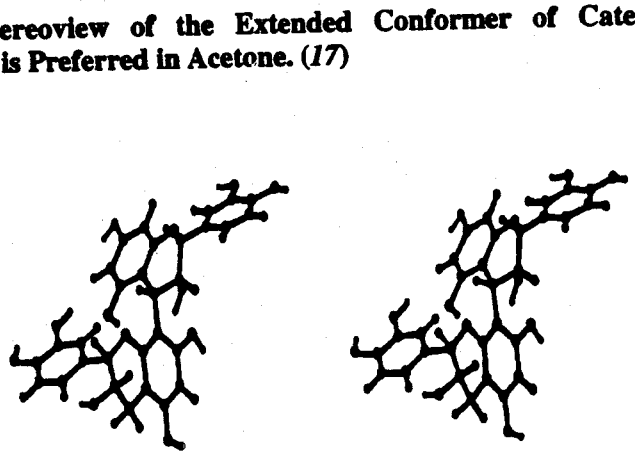
The coupling constants observed for the upper unit heterocyclic ring (C-ring) in both rotational isomers of either catechin-(4α→8)-epicatechin or catechin-(4α→8)-catechin were consistent with a half-chair conformation in which the B-ring was approximately equatorial suggesting that substitution at C-$4_{C\alpha}$ with a bulky flavan unit stabilizes the conformation of the C-ring. (*17*) However, line shape analysis of H-$3_P$ in either rotamer of catechin-(4α→8)-epicatechin suggested that this pyran ring is distorted toward a sofa conformation (Figure 12).

**Figure 12: Lineshape Analysis of H-$3_F$ in the Lower Unit of Procyanidin Dimers Suggests a Sofa Conformation that is Supported by NOESY and Long-Range COSY Experiments. *(17)***



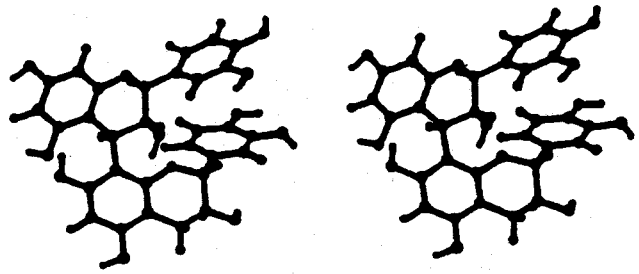

There would not seem to be any way to make a Boltzmann sum of the A- and E-conformer couplings and arrive at the observed splitting for H-$3_F$. NOESY and NOE difference experiments also supported the conclusion that the lower unit heterocyclic ring is distorted toward a sofa conformation. In the extended conformer, NOE is seen between H-$2_E$ and H-$4_C$ while a long range COSY spectrum shows an approximate 90° angle between H-$4_C$ and H-$6_D$. A preferred conformation similar to that shown in Figure 13 is consistent with all these observations.

**Figure 13: Stereoview of the Extended Conformer of Catechin-(4α→)-Epicatechin that is Preferred in Acetone. *(17)***



By contrast, the evidence suggests that $\pi$-$\pi$ interaction between the B- and E-rings stabilizes the conformation shown in Figure 14 for the more compact rotamer that predominates in water.

**Figure 14: Stereoview of the Conpact Conformer of Catechin-(4α→8)-Epicatechin that is Preferred in Water.** *(17)*



**Interaction Between Flavanoids and Oligopeptides**

Haslam (*15*) made an outstanding review of our understanding of the complexation of plant polyphenols with proteins and carbohydrates up to 1987. Murray and Haslam (*25*) studied changes in chemical shifts to show that, in the interactions of pentagalloyl glucose with proline rich peptides, the interaction was selectively with proline and its neighboring residues. In a more recent review, Haslam (*14*) stressed the importance of hydrophobic interactions between plant polyphenols and other biopolymers including proteins or plant polypeptides.

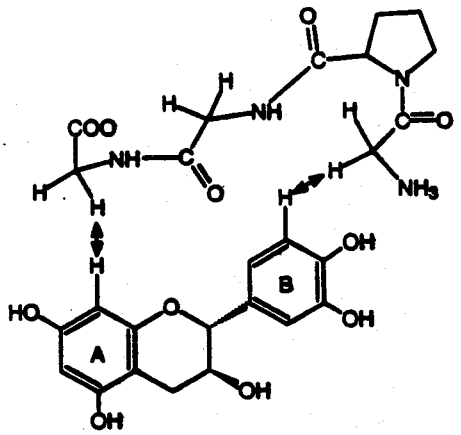
Hatano (*16*) used NOESYHG experiments in an attempt to define the interactions of flavanoids with oligomeric peptides containing prolyl residues where changes in chemical shift were usually too small to measure. Here a challenge was to work with very dilute water or water/$D_2O$ solutions to prevent precipitation of the complex while also suppressing the large water signal. Because of rapid exchange of protons of interest, it was not always possible to work with $D_2O$ as the solvent. We have found the P1-3-3-1 experiment particularly useful in minimizing the large water signals in the proton spectra.

When (+)-catechin and L-proline are combined, the NOESYHG experiment showed correlation between the catechin B-ring protons and the proline Cγ protons. (*16*) However, in combinations of (+)-catechin with the dipeptide Gly-Pro in water, significant cross peaks were seen between the (+)-catechin A-ring protons and the glycine protons. Turning the sequence the other way to combinations with Pro-Gly,

once more a significant cross peak was seen between the catechin H-$8_A$ and glycine protons. The importance of hydrophobic interactions with residues other than proline was further emphasized in NOESYHG experiments with the dipeptides Pro-Val and Pro-PhA. (*16*)

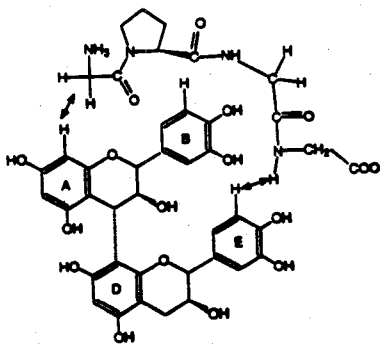
Experiments using the oligopeptides Gly-Pro-Gly-Gly and bradykinin (Arg-Pro-Pro-Gly-PhA-Ser-Pro-PhA-Arg) showed that it is possible to obtain information from NOESYHG experiments at 300 MHz for fairly complex oligopeptides and that conformational properties of the flavan and the oligopeptide are indeed important. (*16*) For example, interactions of (+)-catechin or catechin-(4α→8)-catechin with the tetrapeptide Gly-Pro-Gly-Gly differ. In the interaction with (+)-catechin, H-$8_A$ shows correlation with the $C_\alpha$ methylene protons of the C-terminal Gly unit and a cross peak is also observed between the H-$5_B$ proton and the methylene protons of the N-terminal Gly residue (Figure 15).

**Figure 15: Intermolecular NOE Correlations Between (+)-Catechin And Gly-Pro-Gly-Gly in Water.*(16)***




By contrast, the NOESYHG experiment on the interaction between the dimer catechin- (4α→8)-catechin with the this same tetra-peptide shows correlation of the A-ring H-$8_A$ with the methylene protons of the N-terminal Gly residue while a cross peak is also evident between H-$2_B$ and H-$5_B$ and the amide proton of the C-terminal Gly unit (Figure 16).

**Figure 16: Intermolecular NOE Correlations Between Catechin-(4α→8)-Catechin and Gly-Pro-Gly-Gly in Water.** *(16)*



In all the work done to date, we have not seen significant changes in chemical shifts that suggest strong polar interaction. This is to be expected because the presence of water should disrupt hydrogen bonding. However, we have found that NOESYHG experiments can reveal intermolecular associations in water that emphasize the importance of conformations for both the flavan and peptide as well as their potential for hydrophobic association.

It is important to note that cross peaks representing intermolecular associations between the flavanoids themselves are also prominent in these NOESY experiments. (*16*) Self association of the polyflavanoids is particularly evident when dissolved in water and other flavanoids molecules compete strongly with the polypeptides in the systems we have examined thus far. It is generally considered important that the tannin have a molecular weight equivalent to about 3 to 4 flavanoid units in order to complex strongly to proteins. However, we have found that even addition of the monomeric flavan-3-ol (+)-catechin and in low mole ratios to the number of prolyl units in polyproline results in precipitation. Solubility constraints are at least as important as the problem of working with large HOD resonance in these experiments.

## CONCLUSIONS

Progress in our understanding of the chemistry of condensed tannins has been intimately tied to the development of NMR technology and, fortunately, there seems no end in sight to progress in new NMR instrumentation and experiments. It does not seem long ago that the first $^{13}$C-NMR experiments on polymeric procyanidins were presented simultaneously by Lawrence Porter (*7*) and Karchesy (*23*) at a 1979 ACS symposium organized by J.W. Rowe in Hawaii. The great progress that has been

made since then is largely thanks to the development of NMR instrumentation. This review, by necessity, is focused on providing some examples from our own work and is not meant to be a thorough review of all the NMR experiments that have been so important to advancing plant polyphenol chemistry. We hope the examples and literature cited here will be of some help in providing an understanding where we have been and where we are headed in applying NMR experiments to understanding the chemistry and significance of an important class of plant polyphenols.

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